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Na,K-ATPase expression and cell volume during hypertonic stress in human renal cells

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Na,K-ATPase expression and cell volume during hypertonic stress in human renal cells. Primary cultures of human renal cortex cells were incubated in hypertonic medium and low K^+ medium to determine the effect on Na,K-ATPase α and β subunit expression, cell water, and intracellular ions. Cells exhibited functional characteristics of proximal tubules based on PTH stimulation of cAMP and the presence of Na^+-dependent phosphate transport. When either NaCl or sucrose was added to increase medium osmolality to 500 mOsm/kg, β subunit mRNA increased relative to control between 2.4 and 3.2-fold by six hours, and was still near twofold higher after 24 hours, while α subunit mRNA increased to about 1.5 times control by six hours. In low K^+ medium, only β mRNA increased. Hypertonic incubation increased Na,K-ATPase activity by 39% to 66% after 24 hours. Cell water was 70% of control at one hour, but increased to 90% of control by 24 hours. Only about 40% of the volume regulatory increase depended on accumulation of Na^+ and K^+. These results demonstrate that primary cultures of human proximal tubule cells can respond to hypertonic stress by induction of Na,K-ATPase.

Hypertonic stress initially decreases cell volume and alters intracellular ion concentrations. Cells exposed to a hypertonic environment can respond by a variety of mechanisms, including activation of ion transport systems, gene induction, and accumulation of organic osmolytes [1]. Some cells undergo a regulatory volume increase (RVI). RVI can involve passive water uptake secondary to increased NaCl transport, mediated by Na^+-K^+-$2Cl^-$ cotransport or parallel Na^+-H^+ and Cl^--HCO_3 exchangers [1, 2]. When Na^+ permeability is enhanced by these transport systems, Na,K-ATPase (the sodium pump) may function to exchange intracellular Na^+ for extracellular K^+ to restore normal cellular concentrations following RVI [2]. Na,K-ATPase also maintains proper Na^+ and K^+ concentrations and contributes to normal cell volume regulation and solute absorption.

Na,K-ATPase is a transmembrane protein, located on the basolateral surface of renal tubule cells, and is composed of two main subunits designated α and β. Incubation of kidney cells in low K^+ medium alters intracellular [Na^+] and [K^+] and results in either a coordinate [3] or a noncoordinate [4, 5] increase in Na,K-ATPase α and β subunit mRNA, depending on the cell type. Incubation in hypertonic medium, which decreases cell volume and alters intracellular ion concentration, increases Na,K-ATPase activity and coordinately elevates Na,K-ATPase α and β mRNA levels in Madin-Darby canine kidney (MDCK) cells [6]. However, MDCK cells are a continuous cell line that expresses cellular antigens consistent with a late distal tubule or early collecting tubule origin [7].

To assess whether similar mechanisms are present in human renal cells, we prepared primary cultures of human kidney cortex that exhibited proximal tubule characteristics. Proximal tubule cells absorb solutes coupled to sodium, which results in a passive absorption of water at a rate equivalent to one to four times the cell volume per minute [8]; thus, control of cell volume and intracellular Na^+ and K^+ gradients is essential. We measured the effect of hypertonic stress on the time course and pattern of change in Na,K-ATPase α and β mRNA, Na,K-ATPase activity, cell water content, and intracellular Na^+ and K^+. These parameters were compared for cells incubated in low K^+ medium, which stimulates Na,K-ATPase expression in several other renal cell lines [3, 4, 9]. In both types of media, Na,K-ATPase β mRNA increased, but α subunit mRNA increased less than β in hypertonic media and not at all in low K^+ medium. The magnitude of changes in Na,K-ATPase activity in hypertonic medium more closely tracked changes in α subunit than β subunit mRNA. These results suggest that kidney tubule in cells of human origin can respond to hypertonic challenge by up-regulation of Na,K-ATPase through mechanisms that are inactivated by low K^+ medium.

Methods

Cell culture

Primary cultures of renal tubule cells were initiated using a method that produces predominantly proximal tubule cells [10], modified to increase the yield of viable cells. A normal cortical tissue sample was removed from fresh nephrectomy specimens and placed on ice. The capsule was stripped from a cubic centimeter of tissue and the tissue minced with sterile razor blades. Cells were dissociated in Ca^{2+} and Mg^{2+}-free Hank's balanced saline solution (HBSS) containing 0.63 mg/ml of hyaluronidase type I-S (Sigma) and 1.0 mg/ml collagenase CLS 3 (Worthington Biochemical Corp.). The mixture was stirred for one hour at 37°C. The pH was checked and adjusted if necessary to 7.2. The larger tissue fragments were allowed to settle and the tissue suspension aliquoted into Falcon Primaria T-75

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flasks (Becton-Dickinson). The cells were incubated in Dulbecco's Modified Eagle's (DME) medium and Ham's F-12 medium in a 1:1 mixture containing 15 mM HEPES, 3% fetal calf serum (Gibco), 2.5 μg/ml Fungizone, 2 mM L-glutamine, and 0.08 mg/ml gentamicin. The cells were incubated overnight in a humidified 8% CO_2 incubator at 37°C and then changed to serum-free defined medium. Cells remaining in suspension were transferred to a new flask for another 24 hours to allow additional cells to attach. No difference in morphology or function of these cells was noted compared to cells that attached during the first 24 hours. The serum-free medium was composed of DME/Ham's F-12 (1:1), selenium 5 ng/ml, insulin 5 μg/ml, transferrin 5 μg/ml, hydrocortisone 36 ng/ml, triiodothyronine 4 pg/ml, prostaglandin E_1 25 ng/ml, recombinant human epidermal growth factor (Bachem) 10 ng/ml, L-glutamine 2 mM, and Fungizone 2.5 μg/ml [10]. Medium was changed every three to four days.

Confluent cells were subcultured by incubating in HBSS for 30 minutes and trypsinizing with 0.125% trypsin-0.02% EDTA. The cells were incubated overnight in DME/Ham's F-12 with 3% fetal calf serum to improve adherence to culture dishes, then grown in serum-free defined medium. The cells were passed three to four times at a 1:8 ratio before becoming senescent as evidenced by their failure to grow. Cryopreservation was tolerated using serum-free medium with 10% DMSO as cryoprotectant.

Experimental media

Experimental media were prepared from a custom-formulated low Na^+ base medium similar to DME, except that no NaCl or KCl was added (JRH Biosciences). NaCl was added to adjust the medium [Na^+] to 135 or 223 mM so that the final medium osmolality was either 300 (control medium) or 500 (hypertonic medium) mOsm/kg. In some experiments, sucrose was added to control medium to adjust the osmolality to 500 mOsm/kg; [Na^+] of this medium was the same as control medium (135 mM). KCl was added to the above media so that the final [K^+] was 5 mM. Low K^+ medium was prepared by replacing KCl with NaCl. The final [K^+] of this medium was 0.25 mM as measured by atomic absorption spectrophotometry.

mRNA analysis

Total RNA was isolated using a single-step guanidine thiocyanate method [11]. Briefly, cells were grown to confluence on 100 mm dishes and lysed with 4 M guanidine thiocyanate. The lysate was extracted with water-saturated phenol and chloroform/isoamyl alcohol, washed, and dissolved in 100 μl DEPC-treated water. RNA was quantitated by ultraviolet absorbance at 260 nm. Total RNA (10 μg) was fractionated on a 1% denaturing formaldehyde agarose gel [11]. The RNA was transferred by capillary blotting to nitrocellulose in 10× SSC (where 1× SSC is 150 mM NaCl, 15 mM sodium citrate) and immobilized by UV crosslinking with a Stratagene Stratalinker™.

To quantitate levels of α and β mRNA, blots were probed with ^{32}P-labeled dog kidney α1 cDNA [3] and either dog β1 cDNA [6] or human β1 cDNA probe (prepared as described below). Dog cDNA was labeled using a random-primed method (Boehringer Mannheim). Human β_1 cDNA was labeled with the Klenow fragment of DNA polymerase using a polymerase chain reaction (PCR) primer that resulted in labeled cDNA complementary to the mRNA. The blots were hybridized using either a conventional formamide hybridization solution or Quikhyb™ (Stratagene), a hybridization solution designed to shorten hybridization time. For the first method, hybridization was overnight at 42°C in 50% formamide, 5× SSC, 50 mM sodium phosphate (pH 7.4), 2.5 mM sodium pyrophosphate, 0.2% SDS, 50 μg/ml yeast tRNA, 100 μg/ml salmon sperm DNA, and 0.1% each of ficoll, polyvinylpyrrolidone, and BSA. Blots were washed with 500 ml of 2× SSC/0.1% sodium dodecyl sulfate (SDS) at room temperature for ten minutes followed by a 250 ml wash at 55°C in 0.2× SSC for fifteen minutes. Using Quikhyb™ solution, hybridization was for one hour at 68°C with two room temperature washes in 2× SSC/0.1% SDS for five minutes followed by a 15 minute wash at 60°C in 0.1× SSC/0.1% SDS. Both methods produced similar quantitative results, but the Quikhyb™ method was more rapid and resulted in less background. Filters were exposed to X-ray film at −70°C with intensifying screens. mRNA was quantitated using an LKB/Pharmacia laser densitometer to scan the autoradiogram and calculate the mean peak area from eight scans across the width of the band. The blots were also probed with ^{32}P-labeled Chinese hamster ovary gene B (CHOB) cDNA, which is constitutively expressed in a wide variety of tissues and cell lines [12]. The α and β mRNA peak area were normalized to the CHOB peak area for each lane to control for variability in RNA loading.

β subunit cDNA probe

β subunit mRNA was initially measured with a dog kidney β1 cDNA probe. However, at the stringency employed it was difficult to obtain a hybridization signal sufficiently strong for reliable quantitation. Thus, a human β cDNA probe was synthesized using PCR techniques [13]. The cDNA sequence for the human β subunit of Na,K-ATPase [14] was used to design primers that were within the protein coding region for the β subunit. Primers were synthesized that corresponded to bases 27 to 50 and were complementary to bases 897 to 920 of the human β subunit cDNA. cDNA was synthesized from 20 μg of total RNA, prepared from human proximal tubule primary cultured cells, using 25 units of avian myeloblastosis virus reverse transcriptase (Promega). The β subunit target cDNA segment was amplified by PCR using Taq DNA polymerase (Promega). The reaction was cycled 30 times with annealing at 55°C, elongation at 72°C, and denaturing at 93°C. Gel electrophoresis of the reaction showed a single band 900 base pairs long. Three 1 μl plugs were taken from the band and used as target DNA for three subsequent PCR reactions. These reactions were combined and run on an agarose gel. The resulting single band was cut from the gel and the DNA recovered using an electroeluter (International Biotechnologies, Inc.).

Na,K-ATPase activity

Activity of the Na,K-ATPase was estimated from the ouabain inhibitable fraction of the ATP-phosphohydrolase activity under optimal conditions at 37°C [15]. Human renal cells were grown to confluence in 100 mm dishes, washed three times with ice-cold phosphate buffered saline, scraped, and pelleted at 1600 × *g*. The cell pellet was homogenized at 4°C with a Tissuemizer (Tekmar) at setting 40 for one minute. The sample was centrifuged at 1600 × *g* and the supernatant recovered.

Membranes in the supernatant were permeabilized to ATP with 0.1% deoxycholate. The cell homogenates were incubated with 70 mM NaCl, 12 mM KCl, 10 mM $MgCl_2$, 3.33 mM EDTA, 100 mM Tris (pH 7.8), and 3 mM ATP for 15 minutes at 37°C. The reaction was stopped and the protein precipitated with 5% trichloroacetic acid. The protein was pelleted at 12,800 × *g*. Inorganic phosphate in the supernatant was measured and normalized to the protein content. Na,K-ATPase activity was calculated from the difference between P_i released in the presence and absence of 1 mM ouabain and is expressed as μmol (mg protein)$^{-1}$ hr^{-1}.

Cell water and intracellular cations

To measure cellular water content, cells were incubated with [^{14}C]urea (0.25 to 1 μCi/dish) for at least one hour, then washed four times with ice-cold 120 mM $MgCl_2$. The cells were air-dried then lysed with 1 ml of 5% trichloroacetic acid for one hour. To calculate intracellular water, 750 μl of the lysate was counted in a liquid scintillation counter and divided by medium [^{14}C]urea specific activity (cpm/ml). Cell water content was expressed as μl cell water per mg cell protein content. To measure intracellular [Na^+] and [K^+], cells were washed and dried as above then lysed with a Na^+-free hypotonic alkaline solution containing a nonionic detergent (32 mM NH_4OH, 8 mM CsCl, and 0.003% Acationox [VWR]). The lysate was collected and centrifuged five minutes at 250 × *g*. The Na^+ and K^+ content of the supernatant was determined with an atomic absorption spectrophotometer and is expressed as μmol/mg protein. [Na^+] and [K^+] are expressed as mmol/liter cell water.

cAMP formation

cAMP formation was determined by the production of [^{3}H]cAMP from [^{3}H]ATP using the method of Shimizu [16]. Cells were incubated 30 minutes in DME with 20 mM HEPES and 5 μCi/ml of [^{3}H]adenine (American Radiolabeled Chemicals) to allow [^{3}H]adenine incorporation into intracellular ATP. The cells were washed twice then stimulated for five minutes with either 2 μM PTH (1-34) (Sigma) or 1 μM forskolin (Sigma) in medium containing 1 mM isobutylmethyl-xanthine (IBMX), a phosphodiesterase inhibitor. The medium was removed and the cells lysed with 5% TCA. [^{3}H]cAMP and [^{3}H]ATP were separated sequentially with Dowex and alumina columns. Results were quantified by liquid scintillation counting and are expressed as percent conversion of [^{3}H]ATP into [^{3}H]cAMP.

P_i transport

Total P_i transport was measured in confluent cells. Primary cultures of human proximal tubule cells were grown to confluence and incubated in uptake solution, consisting of 150 mM NaCl, 1.0 mM $CaCl_2$, 1.8 mM $MgSO_4$ and 10 mM HEPES, pH 7.4. Transport was initiated by adding 1 ml of the uptake solution containing 0.1 mM $K_2H^{32}PO_4$ (3 μCi/ml). The cells were incubated 10 minutes at 37°C and the reaction terminated by adding 1 ml of ice-cold uptake solution made with 150 mM choline chloride substituted for NaCl. The cells were washed with the same solution three times and dissolved in 0.2 N NaOH. ^{32}P radioactivity in the solubilized sample was counted on a liquid scintillation counter. The results were expressed as nmol P_i/mg protein/10 minutes of uptake. Na^+-independent P_i uptake was determined in the same way except that choline chloride replaced the NaCl in the uptake solution. Na^+-dependent transport was then calculated from the difference in total and Na^+-independent P_i transport measured in parallel culture dishes.

Statistical analysis

Data are reported as means ± standard error of the mean (SE). The significance of differences in measurements were estimated by analysis of variance (ANOV) with the Super-ANOVA general linear modeling software package (Abacus Concepts, Berkeley, California, USA). Type III sums of squares were used for data with unequal cell frequencies [17]. When differences in the overall model were statistically significant based on the F-ratio ($P < 0.05$), individual means were tested for significant differences. Means in one-way ANOV models were compared by Dunnett's two-tailed procedure for multiple comparisons with control [18]. Means that were combinations of main effects in two-way and three-way ANOV models (such as data from different treatments at different times) were compared by the use of contrasts [19].

Other procedures

Protein measurements were performed with the bicinchoninic acid method [20] using bovine serum albumin as a standard. A Wescor vapor pressure osmometer was used to determine medium osmolality. Unless otherwise indicated, all chemicals used were of reagent grade.

Results

Isolation and characterization of primary cultures of human proximal tubule cells

Cells with minimal fibroblast contamination were recovered from renal cortex specimens by collagenase dissociation and growth in serum-free defined medium. The cells were predominantly epithelioid in appearance and exhibited contact inhibition. Monolayers cultured on plastic dishes formed "domes," typical of ion transporting epithelia. To determine whether these cells expressed characteristics of proximal tubule cells, receptor-coupled stimulation of cAMP production and the presence of Na^+-dependent phosphate transport was assessed. Figure 1 shows the effect of parathyroid hormone (PTH), forskolin, and vasopressin on cAMP production in the presence of IBMX, a phosphodiesterase inhibitor. Treatment with 2 μM PTH, an agonist coupled to adenylyl cyclase, stimulated cAMP production more than fourfold after five minutes and more than sevenfold after 30 minutes. Forskolin (1 μM) directly activated adenylyl cyclase and increased cAMP production after five minutes by an amount comparable to a five minute PTH treatment. In contrast, 1 μM vasopressin, which does not act in the proximal tubule, had no effect on cAMP. As an additional test for proximal tubule characteristics, eight cultures from two independent tissue samples were tested for the presence of Na^+-dependent phosphate transport. In the presence of Na^+, total phosphate transport was 9.96 ± 0.47 nmol/mg protein/10 minutes. When Na^+ in the transport assay medium was replaced with choline, phosphate transport was 1.62 ± 0.51 nmol/mg protein/10 minutes, demonstrating that approximately 84% of phosphate transport in these cells is dependent on extracellular Na^+.

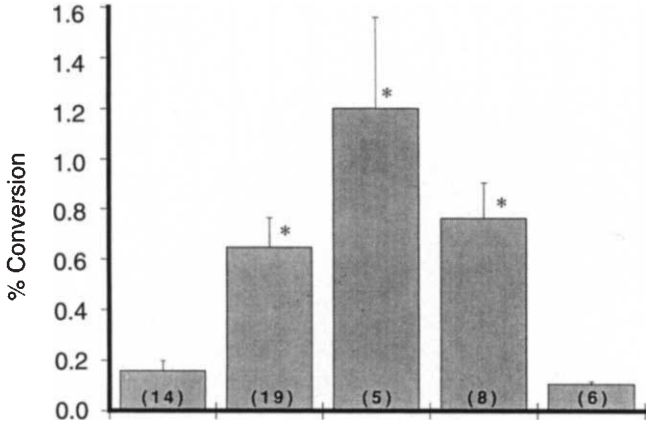

Fig. 1. *Effect of PTH, forskolin, and vasopressin on cAMP production in primary cultures of human kidney cortex.* Production of cAMP was measured after cells were treated with 2 μM PTH (for 5 min or 30 min), 1 μM forskolin (for 5 min), or 1 μM vasopressin (for 5 min). Bars represent means ± SE for the number of measurements indicated in parentheses. * Significantly different from control ($P < 0.05$).

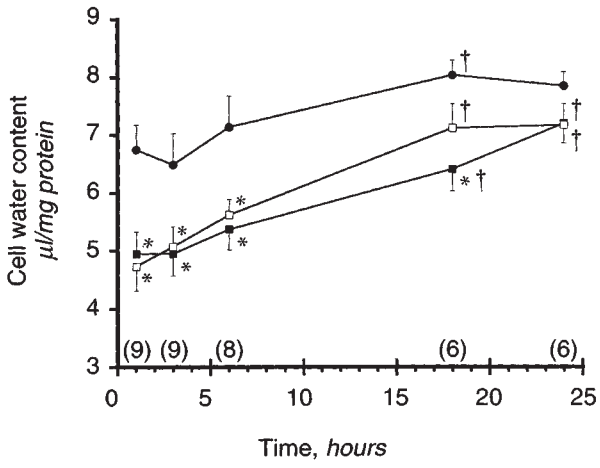

Fig. 2. *Effect of incubation in hypertonic medium on cell water content.* Cells were incubated in control medium (●) and in medium made hypertonic (500 mOsm/kg) by the addition of NaCl (■) or sucrose (□). Cell water was determined from the volume of distribution of [^{14}C]urea after at least one hour of equilibration. Data shown are the mean ± SE and the number of separate measurements at each time are shown in parentheses. * Indicates a significant difference relative to the control value at the same time ($P < 0.05$); † Indicates a significant difference from the one hour value in the same medium ($P < 0.05$).

Alterations in cell water during incubation in hypertonic medium

To determine the effect of hypertonicity on cell water content, cells were grown to confluence on 35 mm dishes and incubated from one to 24 hours in hypertonic medium. Medium osmolality was raised to 500 mOsm/kg by adding either NaCl or sucrose to control medium. Cell water was determined from the distribution of [^{14}C]urea following at least one hour of equilibration. Cell water in control cells gradually increased over the course of the experiment. The water content of cells incubated in hypertonic medium with added NaCl fell to 73% of control at one hour then increased to 92% of the control cell water content by 24 hours (Fig. 2), representing a significant regulatory volume increase. The response of cells exposed to sucrose-containing hypertonic medium was similar, falling to 70% of control at one hour and returning to 91% of control at 24 hours. The cell water content of cells incubated in low K^+ medium was not significantly different from control values at any time point and is omitted from Figure 2 for clarity.

Changes in intracellular ion concentration and content during incubation in hypertonic medium

To determine whether changes in cellular Na^+ and K^+ contributed to the increase in cell water content, the time course of changes in the cellular content and concentration of these ions was examined. Ion content was measured by atomic absorption spectroscopy and expressed as μmol/mg cell protein. Intracellular ion concentrations were calculated using estimates of cell water from identically-treated cells. Control cell [K^+] did not change significantly during the experiment (Fig. 3A). As would be expected from the loss of cell water, cells incubated in hypertonic media for one hour had an elevated [K^+], which decreased with time as cell water increased. In contrast, cell K^+ content in control cells and cells in hypertonic media were not significantly different (Fig. 3B). For comparison, both K^+ content and [K^+] were depressed in cells incubated in low K^+ medium, which inhibits Na,K-ATPase activity and dissipates transmembrane ion gradients.

Cell Na^+ in hypertonic media generally followed the pattern shown by cell K^+ during the first six hours of incubation. Except for a consistent drop at three hours control cell Na^+ content and [Na^+] were relatively stable (Fig. 3 C and D). The [Na^+] of cells in hypertonic media was elevated at one hour and in both hypertonic media cell [Na^+] fell by six hours. Subsequently, cells in sucrose hypertonic medium returned to the same intracellular [Na^+] as control cells, but the [Na^+] of cells in hypertonic medium made by adding NaCl was significantly elevated above control values throughout the 24 hour incubation. There was no significant change in total Na^+ content of cells in hypertonic medium as a function of time, although cells in hypertonic medium with added NaCl had a Na^+ content that was higher than controls. In low K^+ medium, the Na^+ content and [Na^+] of cells were significantly higher than control cells throughout the incubation period.

To assess whether the changes in cell Na^+ and K^+ content could account for the observed recovery of cell water in cells incubated in hypertonic media, the change in water content between one hour and 24 hours was compared to the total content of Na^+ plus K^+ (the major intracellular cations) at the corresponding times. As shown in Table 1, the water content of control cells increased over the course of the experiment, but the change was not statistically significant at 24 hours. The water content of cells in hypertonic media was significantly lower than control at one hour and increased by 24 hours. There was no difference in cation content of cells in hypertonic media compared to control at either 1 hour or 24 hours, but there was a significant increase in all media at 24 hours compared to one

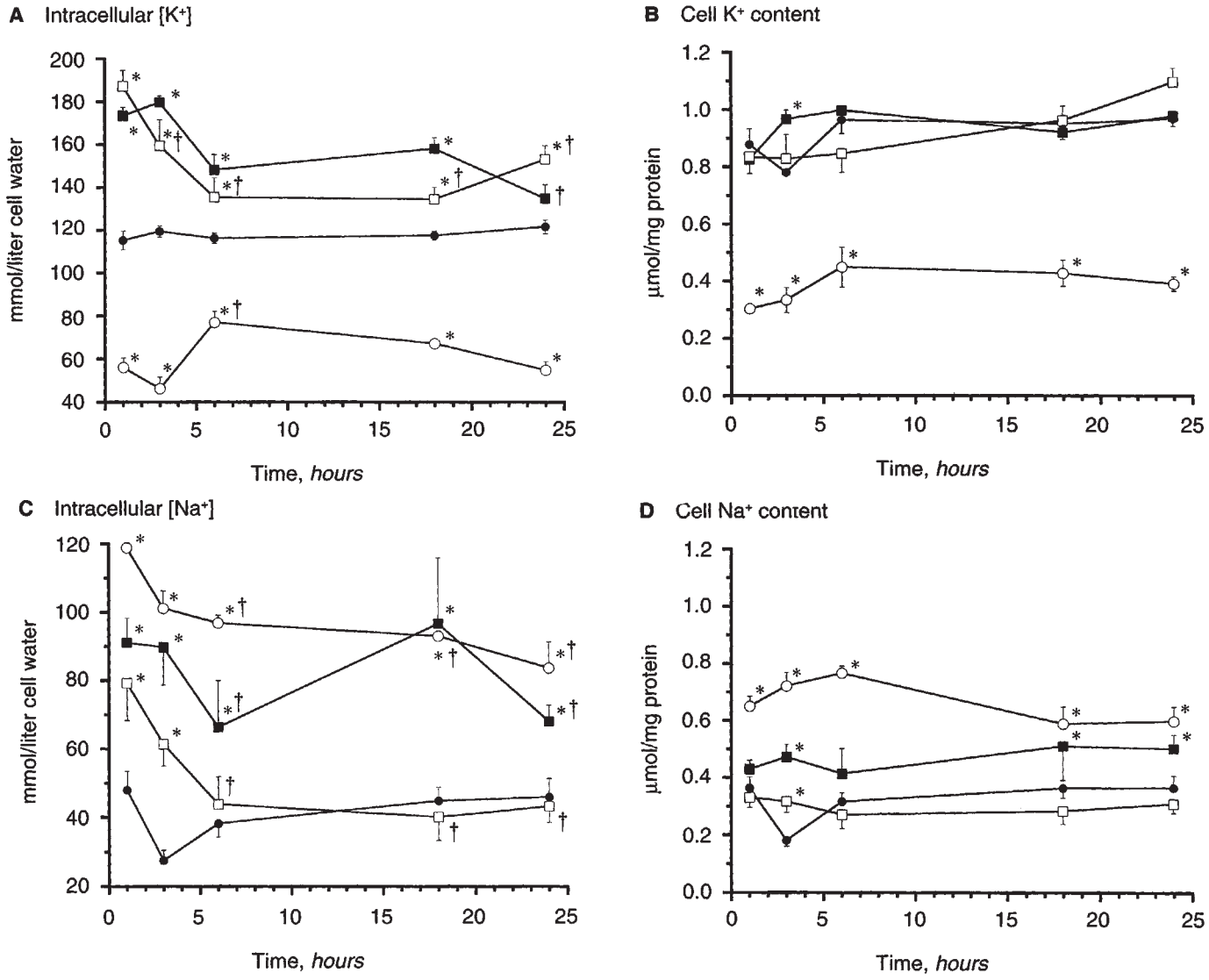

Fig. 3. *Effect of incubation in control, hypertonic, or low K^+ medium on intracellular Na^+ and K^+ content and concentration.* Cells were incubated in control medium (300 mOsm/kg; ●), in medium made hypertonic (500 mOsm/kg) by the addition of NaCl (■) or sucrose (□), or in low K^+ medium (○). Concentration was calculated using cell water measured in parallel dishes. Data shown are the mean ± SE of six independent samples. * Indicates a significant difference from control at the same time point ($P < 0.05$); † Indicates a significant difference from the 1 hr value in the same medium ($P < 0.05$).

Table 1. Comparison of changes in cell water with intracellular ($Na^+ + K^+$) content in control and hypertonic media

Incubation medium	Cell water content *µl/mg*			Cell ($Na^+ + K^+$) content *µmol/mg*		
	1 Hr	24 Hr	Change	1 Hr	24 Hr	Change
Control (300 mOsml/kg)	6.75 ± 0.43	7.85 ± 0.24	16%	1.24 ± 0.06	1.33 ± 0.06	7%
500 mOsml/kg (NaCl added)	4.95 ± 0.38[a]	7.20 ± 0.34[b]	45%	1.25 ± 0.06	1.48 ± 0.04[b]	18%
500 mOsml/kg (sucrose added)	4.72 ± 0.42[a]	7.17 ± 0.31[b]	52%	1.14 ± 0.08	1.40 ± 0.02[b]	23%

Cell ($Na^+ + K^+$) content was calculated from data in Fig. 3 B and D. Percentage values are change from the 1 hr measurement.
[a] Significantly different from control at the same time ($P < 0.05$)
[b] Significantly different from 1 hr in the same medium ($P < 0.05$)

hour. Less than half of the measured increase in cell water can be accounted for by water osmotically obligated due to the change in cell ion content. This suggests that intracellular osmolytes other than Na^+ or K^+ may have a role in the recovery of cell volume in these cells.

Na,K-ATPase activity following incubation in hypertonic medium

Na,K-ATPase activity of cell homogenates was measured to determine whether incubation in hypertonic medium altered

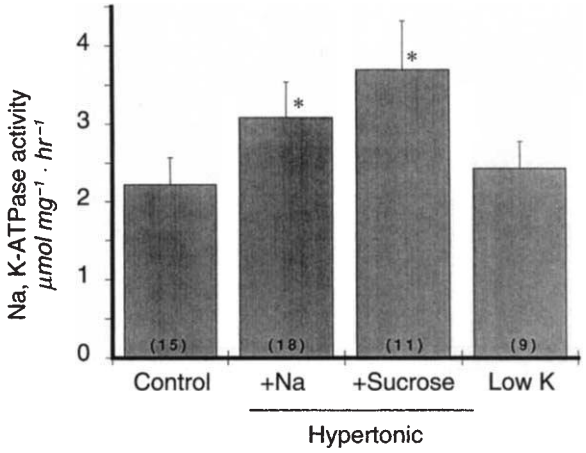

Fig. 4. *Effect of hypertonic stress and K^+-depletion on Na,K-ATPase activity.* Na,K-ATPase activity was measured in cell homogenates after incubation for 24 hours in control medium, hypertonic medium (500 mOsm/kg) made by adding either NaCl or sucrose, or low K^+ medium. The bars represent means ± SE of the number of measurements indicated in parentheses. * Indicates a significant difference from control ($P < 0.05$).

functional cation transport capacity. Na,K-ATPase was determined from the ouabain-inhibitable fraction of the ATP-phosphohydrolase activity under optimal conditions. Assays were performed after incubation in hypertonic media for 24 hours. As shown in Figure 4, incubation in hypertonic medium significantly increased Na,K-ATPase activity by 39% in hypertonic medium with added NaCl and 66% in hypertonic medium containing sucrose. In contrast, incubation of cells in low K^+ medium for 24 hours did not change Na,K-ATPase activity relative to control.

Concentration of Na,K-ATPase α and β subunit mRNA during incubation in hypertonic medium

To investigate whether the increase in Na,K-ATPase activity resulting from hypertonic stress might involve stimulation of Na,K-ATPase expression, the concentration of α and β subunit mRNA were measured by Northern blot analysis. Cells were grown to confluence after one to three passages from twelve different tissue samples and incubated in experimental medium for one to 24 hours. A dog kidney α1 subunit cDNA probe detected a single mRNA species that was 4.6 kilobases (kb) in size (Fig. 5). A human kidney β subunit cDNA probe, prepared by PCR, hybridized to a single mRNA that was 3.1 kb in size. Figure 6A summarizes the results obtained by quantitating changes in α and β subunit mRNA using laser densitometry. Incubation of cells in hypertonic medium (made by adding NaCl) elevated both α and β subunit mRNA, but the β subunit increase was significantly higher than the α subunit at 3 hours, 6 hours, and 18 hours. To investigate whether stimulation of Na,K-ATPase mRNA by hypertonic medium required an increase in extracellular Na^+, cells were incubated in sucrose-containing hypertonic medium. Figure 6B demonstrates that when sucrose was the added osmolyte, mRNA for both subunits increased, although β subunit mRNA was significantly higher than α subunit mRNA at 6 hours and 18 hours. For comparison with conditions that have been previously reported to stimulate Na,K-ATPase mRNA in kidney, cells were K^+-depleted by incubation in low K^+ medium. Under these conditions (Fig. 6C), β subunit mRNA increased significantly but α subunit mRNA did not.

Since mRNA concentration can rise as the result of either an increase in the rate of transcription or a decrease in the rate of degradation, the effect of the mRNA transcription inhibitor actinomycin D was examined. Figure 7 shows that when cells were incubated in hypertonic medium (with NaCl added) for six hours and simultaneously treated with 10 μg/ml actinomycin D, stimulation of α subunit mRNA was inhibited by 83% and stimulation of β subunit mRNA was inhibited by 78%. Thus, most of the increase in Na,K-ATPase α and β mRNA levels caused by hypertonic stress can be accounted for by stimulation of transcription.

Discussion

Our findings demonstrate that hypertonic stress and low K^+ medium both upregulate Na,K-ATPase in primary cultures of human renal cortex. Hypertonicity increased the mRNA concentration of Na,K-ATPase α and β subunits by stimulation of transcription, and resulted in a functional change manifested as an elevation of Na,K-ATPase activity after 24 hours. Although Na,K-ATPase up-regulation by alterations in cell ion concentration has been reported for a variety of cell lines [3–5, 21], and regulation by hypertonic stress was found in a dog kidney continuous cell line [6], to our knowledge this is the first such report for cells of human origin.

The human kidney primary cultures we isolated exhibited functional characteristics of proximal tubule cells. Production of cAMP was stimulated by PTH, a proximal tubule cell marker, and not by vasopressin, which is associated with the distal nephron. The magnitude of the PTH stimulation was higher than the 2.7-fold increase reported for mouse proximal tubule cultures [22], although it was less than the 10- to 35-fold stimulation found in prior characterizations of human proximal tubule cell cultures [23–25]. We found that 84% of phosphate transport was Na^+-dependent, which is comparable to previous reports that phosphate transport is 85% Na^+-dependent in human proximal tubule cells [24]. In addition, the cells formed domes when confluent, a characteristic of ion-transporting epithelia [10, 22]. Finally, it has been shown that human cells isolated from kidney cortex and grown in serum-free defined medium by the methods we employed are histochemically similar to proximal tubule cells [10, 23, 26]. While obviously not identical to *in situ* proximal tubule cells, the primary cultures we isolated allowed experiments that would not have been possible with other renal preparations.

Unlike many established cell lines [27], we found that human renal cortex cells underwent a regulatory volume increase (RVI) during exposure to hypertonic medium. A portion of the increase in cell water content can be explained by an increase in cell ion content. Our measurements of intracellular Na^+ and K^+ were comparable to values previously reported for LLC-PK_1 cells, which are of proximal tubule origin. Control intracellular [Na^+] was about 40 mM and intracellular [K^+] remained near 115 mM. In LLC-PK_1 cells, reported control intracellular [Na^+] ranges from 22 to 40 mM, and [K^+] ranges from 80 to 174 mM [4, 28]. During incubation of LLC-PK_1 cells in hypertonic medium, intracellular [Na^+] increases by 47% and intracellular [K^+] increases by 9% [28]. This agrees well with our finding that,

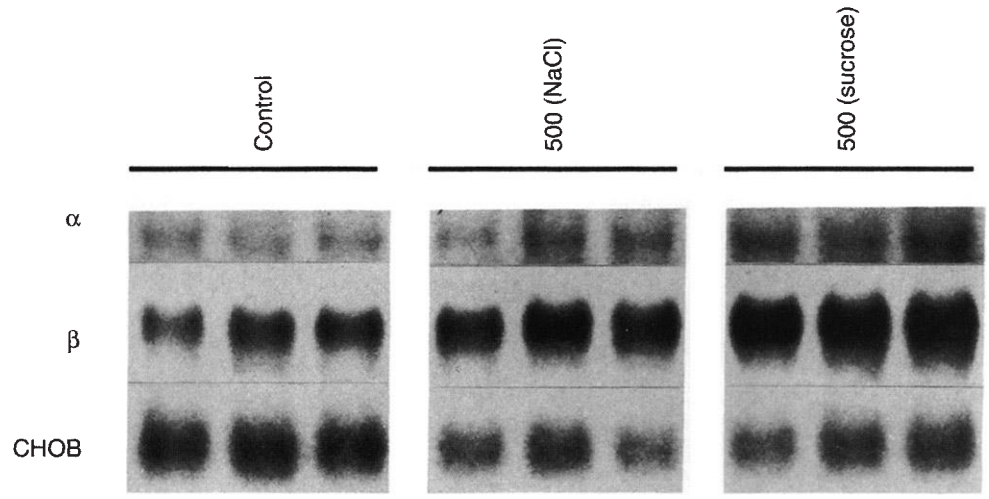

Fig. 5. *Northern blot of Na,K-ATPase α and β subunit mRNA of cells incubated in hypertonic media for 24 hours.* Filters were hybridized to ^{32}P-labeled dog α_1, human β_1, and CHOB cDNA, washed at high stringency, and exposed to film. An autoradiogram of the filter is shown with RNA samples prepared from three cell cultures incubated in each medium. CHOB was used as a gel loading control. Sizes of bands are: α, 4.6 kb; β, 3.1 kb; CHOB, 1.0 kb.

after 24 hours of incubation in hypertonic medium with NaCl added, [Na^+] increased 48% and [K^+] increased 11% compared to control. However, an increase in Na^+ and K^+ content cannot account entirely for the RVI, as shown in Table 1. The increase in cell water may also involve accumulation, by uptake or synthesis, of organic osmolytes such as glycerophosphorylcholine, *myo*-inositol, or betaine [28–30].

Attempts to investigate the role of Na,K-ATPase upregulation in RVI are complicated by the relationship between cell volume, cell ion concentration, and membrane permeability. Hypertonic stress acutely decreases cell water content and can lead to regulatory alterations in membrane ion permeability by activation of ion transport systems such as Na^+-K^+-$2Cl^-$ cotransporters or parallel Na^+-H^+ and Cl^--HCO_3^- exchangers [1]. It is known that a variety of treatments that alter intracellular Na^+ or K^+ can influence Na,K-ATPase expression [3–5, 21]. Inhibition of Na,K-ATPase itself has this effect, as shown by the stimulation of Na,K-ATPase mRNA when cells are incubated in low K^+ medium (Fig. 6C). This issue is further obscured by the role of Na,K-ATPase in counteracting swelling in isotonic medium caused by the high intracellular concentration of negatively charged macromolecules [1]. It has been suggested that an important function of Na,K-ATPase during regulatory volume changes is to restore the high K^+, low Na^+ intracellular environment that could otherwise be dissipated by activation of membrane ion transport [2].

Depending on cell type and conditions, regulation of Na,K-ATPase can involve changes in α and β subunit mRNA that are either coordinate or noncoordinate with respect to time course and magnitude. We found that incubation of primary cultures of human renal cortex cells in hypertonic medium increased both α and β subunit mRNA, but in a noncoordinate manner such that β mRNA increased more than α subunit mRNA at early time points. Incubation in low K^+ medium resulted in a significant rise only in β mRNA levels. This is similar to other non-coordinate regulatory patterns in: (1) cultured chick skeletal muscle, where veratridine increases β subunit mRNA more than α mRNA [21]; (2) primary cultures of rat proximal tubule, where K^+-depletion induces a β mRNA increase that is more rapid and higher than for α mRNA [9]; and, (3) LLC-PK_1 cells, where low K^+ medium increases β mRNA alone with no rise in α mRNA [4]. In contrast, when MDCK cells are exposed to either hypertonic or low K^+ medium, α and β mRNA increases coordinately in both time and magnitude [3, 6]. The mechanisms that give rise to these variations in patterns of subunit expression are not understood. It is now clear that both subunits are required for correct assembly of Na,K-ATPase into an active form [31]. One suggestion has been that there are differences in stability of the two subunits such that β subunit protein is more rapidly degraded, a situation that may dictate a higher rate of β subunit protein synthesis [9].

The signal or signals that lead to an increase in α and β subunit mRNA have been associated with changes in cellular ion content [5]. Veratridine, which activates Na^+ channels and increases intracellular Na^+ concentration, elevates α and β mRNA levels in cultures of chick skeletal muscle [21]. Incubation of MDCK and LLC-PK_1 in low K^+ medium inhibits the sodium pump and increases intracellular [Na^+] while [K^+] falls, resulting in an increase in mRNA for Na,K-ATPase, even at times when intracellular Na^+ increases by only 20% [3, 4]. We found that incubation of human renal cortex cells in hypertonic medium changed the Na^+ and K^+ content only slightly, but did increase the concentration of these ions secondary to a decrease in cell water (Fig. 3). Incubation in low K^+ medium increased intracellular [Na^+] and Na^+ content, and the increase in [Na^+] preceded the increase in β mRNA, but there was no change in α subunit mRNA. However, low K^+ medium causes much larger changes in cell Na^+ and K^+ than hypertonic medium. This difference may affect the mechanisms that regulate Na,K-ATPase subunit mRNA expression. The signal for induction of the aldose reductase gene in PAP-HT25 cells has been proposed to involve an increase in the combined concentration of Na^+ plus K^+ rather than either alone [32]. While our findings are consistent with a role for intracellular Na^+ and K^+ as signals, they do not rule out the possibility that other signals

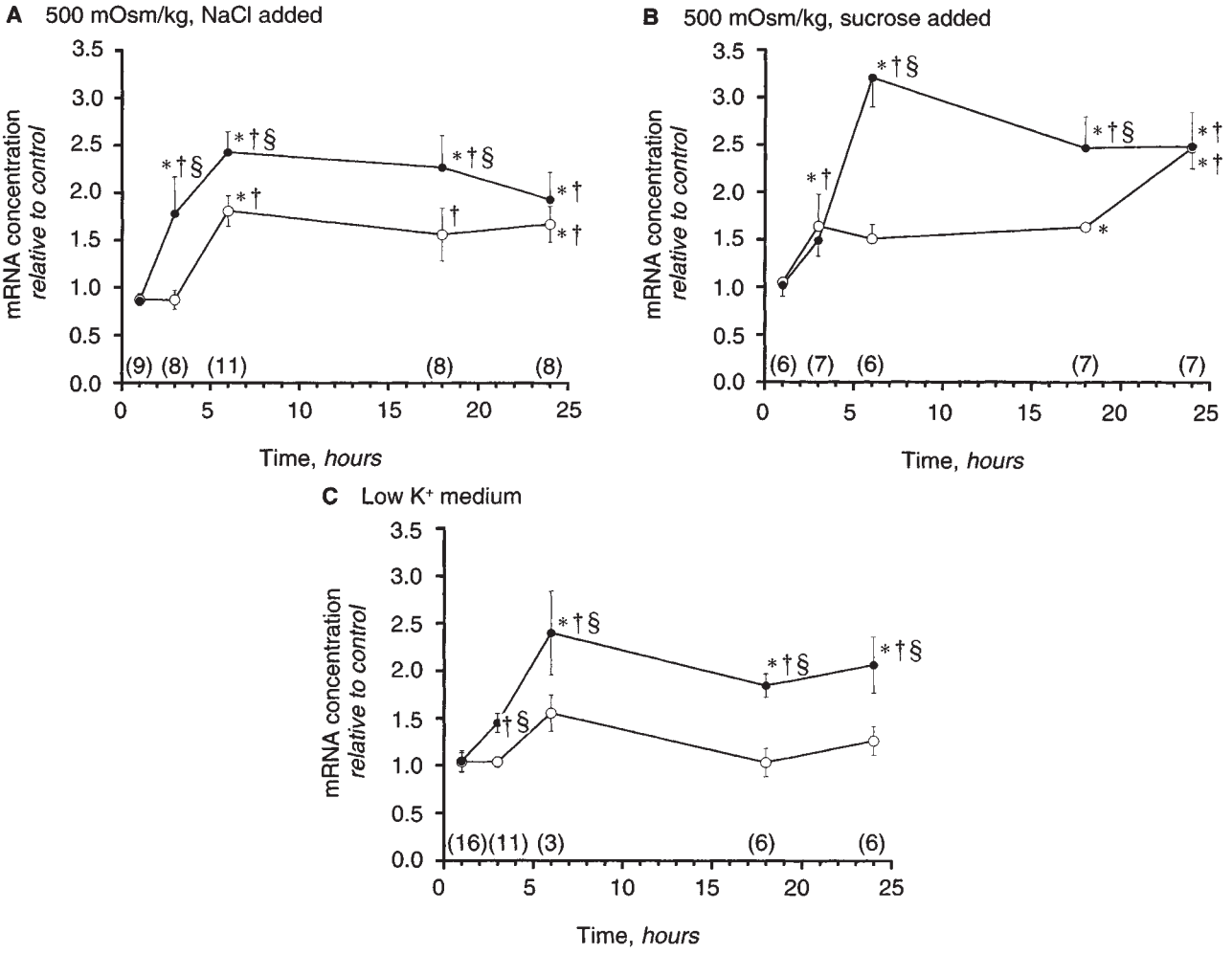

Fig. 6. *Effect of incubation in hypertonic or low K^+ medium on Na,K-ATPase α and β subunit mRNA.* Autoradiograms obtained from Northern blots of α (○) and β (●) subunit mRNA were quantitated by densitometry and values expressed relative to control. Data shown are the mean ± SE and the number of measurements at each time point is shown in parentheses. * Indicates a significant difference from control at the same time point ($P < 0.05$); † Indicates a significant difference from the one hour value in the same medium ($P < 0.05$); § Indicates that β subunit mRNA was significantly different from α subunit mRNA at the same time point ($P < 0.05$).

contribute to these responses. Other candidates include Na^+-coupled processes such as changes in cell pH [33] or changes related to cell volume *per se* [34, 35].

Cellular Na,K-ATPase activity increased when cells were incubated in hypertonic medium for 24 hours. The assay was performed by incubating cell homogenates under optimal conditions with respect to all reactants, suggesting that there was an increase in the cell content of functional Na,K-ATPase. However, it is also possible that Na,K-ATPase (either existing or newly synthesized) had a higher V_{max} than under isotonic conditions. The change in Na,K-ATPase activity we measured in cells incubated in hypertonic medium is similar to the 1.4-fold increase in LLC-PK_1 cells exposed to low K^+ medium [4]. However, in contrast to LLC-PK_1 cells, incubation of our cells in low K^+ medium did not result in a significant increase in Na,K-ATPase activity. This finding is similar to that in K^+-depleted MDCK cells, in which there was no change in Na,K-ATPase activity, in spite of an increase in mRNA, rate of synthesis, and abundance of subunits [3]. The increase in Na,K-ATPase activity in hypertonic medium with either NaCl

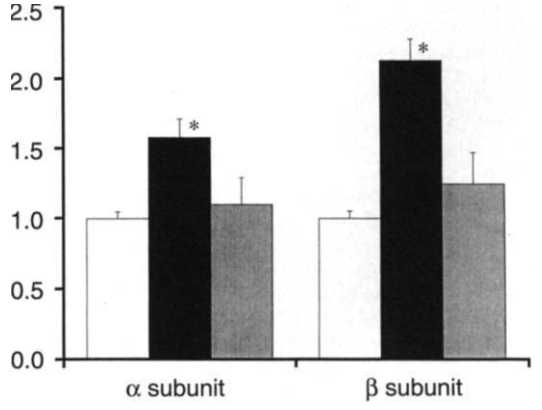

Fig. 7. *Effect of inhibition of transcription on hypertonic stimulation of Na,K-ATPase α and β subunit mRNA.* Cells were incubated six hours in control medium (open bars), hypertonic medium (NaCl added, solid bars), or hypertonic medium containing 10 μM actinomycin D (shaded bars). Values are means ± SE of 10 measurements. * Significantly different from control ($P < 0.05$).

or sucrose added (39% and 66%, respectively) was closer in magnitude to the increase in α subunit mRNA than to the change in β subunit mRNA (Fig. 3). This may signify that the increase in α mRNA is a limiting factor for the increase in Na,K-ATPase activity. This view is also consistent with the inability of low K^+ medium to increase Na,K-ATPase activity (Fig. 4).

Hypertonic stress has recently been observed to induce expression of the immediate early genes *Egr*-l and c-*fos* and the stress protein HSP70 in MDCK cells [36]. The aldose reductase gene is induced in the PAP-HT25 rabbit inner medullary cell line in response to hypertonic stress, and the result is an increase in intracellular sorbitol content [32]. The upregulation of Na,K-ATPase in response to hypertonicity may represent one facet of a more generalized stress response to anisosmotic conditions that enables cells to cope with changes in cell cation concentrations by means of enhanced transport capacity.

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